enterotoxin adsorbent to adsorb and remove the enterotoxin,

said adsorbent comprising a compound with a log P, in which P respresents a partition coefficient in an octanol-water system, value of not less than 2.50 as immobilized on a water-insoluble carrier.

REMARKS

Non-elected claims 1-3 and 5 have been canceled without prejudice or disclaimer to filing in a divisional application. Claim 4 has been amended to more particularly point out and distinctly claim the subject matter of the present invention. Therefore, upon entry of this amendment, claims 4, 6 and 7 remain pending in the present application.

Attached hereto is a marked-up version of the changes made to claim 4 by the instant amendment. The attached page is entitled "**Version with markings to show changes made.**"

Objection to Specification

The Examiner has objected to the specification as containing brackets "[]" throughout and has requested Applicants to submit a substitute specification with the brackets removed. Because the Examiner has requested the substitute specification, Applicants are treating this request as being made under 37 C.F.R. §1.125(a) (see MPEP §608.01(q)).

In accordance with the Examiner's request, Applicants submit herewith a substitute specification. The substitute specification merely removes brackets "[]" at p. 5, line 19-20; p. 7, line 35-p. 8, line 1; p. 14, lines 6 and 28; p. 16, lines 4, 19 and 29; and p. 17, line 4. Therefore, no new matter has been added. Because the Patent Office has requested the substitute specification, it is believed that a marked-up copy showing additions/deletions is not required.

Entry of the substitute specification and withdrawal of this objection are respectfully requested.

Rejection of Claims 4, 6 and 7 Under 35 U.S.C. §112 2nd paragraph

Claims 4, 6 and 7 are rejected under 35 U.S.C. §112 for allegedly failing to

particularly point out and distinctly claim the subject matter which applicants regard as their invention. Claims 4 has been amended to specify that the enterotoxin-containing body fluid is contacted with an enterotoxin adsorbent "to adsorb and remove the enterotoxin." Thus, as requested by the Examiner, claim 4 now positively recites a step of removing the enterotoxin.

Support for this amendment can be found in the specification on page 12, lines 14-16. Therefore, this amendment introduces no new matter. Applicants respectfully submit that claim 4 is not indefinite under 35 U.S.C. § 112 and respectfully request withdrawal of this rejection. Because claims 6 and 7 depend from claim 4, Applicants respectfully submit that claims 6 and 7 are not indefinite for at least the same reasons as claim 4.

Rejection of Claims 4, 6 and 7 Under 35 U.S.C. §102(a)

Claims 4, 6 and 7 are rejected under 35 U.S.C. 102(a) as allegedly being anticipated by EP 0 993 834 A1 to Hirai et al. ("Hirai"). According to the Office Action, the method of Hirai appears to be the same as the method of claim 4 of the present application. Applicants respectfully traverse this rejection. The method of Hirai is directed to a method for removing TSST-1, the molecular weight of which is about 22,000 (See Exhibit A, page 101, table 2-13 and partial translation thereof attached herewith)(Takeshi HONDA, Bioscience Series, "Disease and Baceteriotoxin", pulished by Kagaku Donin Ltd., January 25, 1994). The method of claim 4, in contrast, is directed to a method for removing enterotoxin, the molecular weight of which is about 26,000 to 28,000 (See Exhibit A, page 101, table 2-13; and the specification of the subject application at p. 4, lines 24-26). Therefore, it is respectfully submitted that the method of claim 4 is not anticipated by Hirai.

In addition, TSST-1 and enterotoxin are substantially different in terms of chemical structure. Indeed TSST-1 and enterotoxin lack substantial homology (See Exhibit A, page 103, lines 3-5 and partial translation thereof attached herewith). Furthermore, Hirai does not teach or suggest any methods for removing enterotoxin. Thus, Hirai does not provide motivation to remove enterotoxin using the adsorbents of claim 4. Applicants therefore submit that the method of claim 4 is not obvious in view of Hirai and that claim 4 is allowable. For at least the reasons stated above, Applicants respectfully request withdrawal of the present rejection. Applicants respectfully submit that claims 6 and 7, which depend

from claim 4, are allowable for at least the same reasons as claim 4.

Claims 4, 6 and 7 are also rejected under 35 U.S.C. §102(b) as allegedly being anticipated by the *Journal of Medical Microbiology*, May 1993, 38(5): 354-359 to Nagaki et al. ("Nagaki"). According to the Office Action, "characteristics such as partition coefficient in an octanol water system and a value of not less than 2.50 when immobilized, would be inherent in the material of the prior art." Applicants respectfully traverse this rejection. None of the adsorbents according to Nagaki comprise a compound of log P ≥2.5. For example, neither hydroxyethylmethacrylate polymer-coated activated charcoal (DHP-1) nor acryl ester polymer (XAD-7) comprises a compound immobilized on a water-insoluble carrier. Quaternary ammonium group-introduced styrene-divinylbenzene copolymer (Dowex-7) has a quaternary ammonium group as a ligand. Since $\sum f$ (=log P, see specification page 4, line 30-page 5, line 31) of the quaternary ammonium group is less than 0.675 (in other words, $\sum f$ value of a tertiary ammonium group), Dowex-7 does not comprises a compound of log P ≥2.5 as immobilized on a water-insoluble carrier. Therefore, the compound utilized in the method of claim 4 differs in structure from the compound utilized in the method of Nagaki and therefore the claim 4 is not anticipated by Nagaki.

Furthermore, Nagaki does not teach or suggest the adsorbent comprising a compound of log P ≥2.5 as immobilized on a water-insoluble carrier. Moreover, the method of claim 4 shows an enterotoxin adsorption of ≥65% (See Specification, Table 1, page 17). Enterotoxin adsorption by the adsorbents disclosed in Nagaki only show adsorption ≤50%. Therefore, it is respectfully submitted that claim 4 would not have been obvious over Nagaki. For at least the reasons stated above, Applicants respectfully request withdrawal of this rejection. Applicants respectfully submit that claims 6 and 7, which depend from claim 4, are allowable for at least the same reasons as claim 4.

CONCLUSION

It is respectfully submitted that the present application is now in condition for allowance, which action is respectfully requested. The Examiner is invited to contact Applicants' representative to discuss any issue that would expedite allowance of the subject application.

It is not believed that any extensions of time or other fees are required in connection

with the filing of this response. However, if any fees for extension(s) of time or additional fees are required in connection with the filing of this response, such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and the Commissioner is authorized to charge any such required fees or to credit any overpayment to Kenyon & Kenyon's Deposit Account No. 11-0600.

Respectfully submitted,

KENYON & KENYON

Dated: June 10, 2002

By: Neil M. McCarthy
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Version with markings to show changes made

In the claims:

Claim 4 has been amended as follows:

4. (Twice Amended) A method for adsorptive removal of an enterotoxin in a body fluid which comprises contacting an enterotoxin-containing body fluid with an enterotoxin adsorbent to adsorb and remove the enterotoxin,

said adsorbent comprising a compound with a log P, in which P respresents a partition coefficient in an octanol-water system, value of not less than 2.50 as immobilized on a water-insoluble carrier.

EXHIBITE A

EXHIBIT A

Bioscience Series

生命現象への化学的アプローチ

病気と細菌毒素

本田　武司　著

化学同人

BS シリーズ

生命現象への化学的アプローチ

病気と細菌毒素

本田武司　著

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化)された抗原フラグメントがMHC(major histocompatibility complex)のクラスII分子のポケット部に結合した形でA細胞表面に提示される．これを特定のT細胞クローンのTCR(T cell receptor)のα鎖とβ鎖が共同して認識してT細胞が活性化され，免疫反応がすすんでゆく．

ところで，スーパー抗原とは何だろうか．これは，抗原(外毒素)がフラグメント化されずにA細胞上のMHCクラスII分子(α鎖？)に直接結合する．さらに，これがT細胞上のTCRに認識され，T細胞が活性化される(図2-38)[64]．この際TCRのVβ領域で抗原が認識されるのだが，クローンのワクを超えてVβを表現するT細胞の集団を一括して活性化するのである．たとえば，ヒトでの末梢T細胞は$CD4^+$と$CD8^+$T細胞に大別されるが，スーパー抗原では両細胞とも活性化することができる．このように，スーパー抗原は一般の抗原と次の点で大きく異なる．1)プロセシングを受けることなくA細胞上のMHCクラスII分子に直接結合する，2)MHCクラスII分子(α鎖とβ鎖)に結合した毒素が特定のVβを表現するほとんどすべてのT細胞集団に認識され，T細胞の活性化(TNF-β，IL-2，IFN-γなどのサイトカインの産生)をひき起こす[64]．

12.2 スーパー抗原の種類と性状

以前(1973年ごろ)から，マウスである種の内在性抗原が存在し，これらがいわゆる「スーパー抗原」として働くことが知られていた．しかし最近になって，これらの内在性スーパー抗原は，乳ガンと関係するレトロウイルスの遺伝子産物であることが明らかにされた[65]．したがって，現在ではスーパー抗原は，細菌(毒素)性スーパー抗原とウイルス性スーパー抗原がある．細菌毒素性スーパー抗原について，表2-13にまとめて示した[66]．

これらの毒素は，いずれもタンパク質であり，分子量は20～30kDaである．ブドウ球菌エンテロトキシン(staphylococcal enterotoxins：SE)は別項で述べたが，アミノ酸一次構造上相同性を認めるA～Eの5種類が知られている(SECはさらに三つに細分されるので計7種類)．SEはブドウ球菌性食中毒の原因毒素と考えられている．TSST-1もブドウ球菌が産生し，

表2-13 スーパー抗原活性を示す細菌外毒素の分子量と病原性

	外毒素	産生菌名	分子量	病原性
(1)	毒素性ショック症候群外毒素-1 toxic shock syndrome toxin-1 (TSST-1)	黄色ブドウ球菌	22,049	毒素性ショック症候群(TSS)
(2)	ブドウ球菌腸管毒素A：staphylococcal enterotoxin A (SEA)	〃	27,079	TSS，食中毒
(3)	SEB	〃	28,494	TSS，食中毒
(4)	SEC_1	〃	27,500	TSS，食中毒
(5)	SEC_2	〃	34,000	TSS，食中毒
(6)	SEC_3	〃	27,000	TSS，食中毒
(7)	SED	〃	27,300	
(8)	SEE	〃	26,425	
(9)	表皮剝脱毒 exfoliative toxin A (ETA)	〃	26,951	熱傷様皮膚症候群
(10)	ETB	〃	27,318	熱傷様皮膚症候群
(11)	レンサ球菌発熱性外毒素A streptococcal pyrogenic exotoxin A (SPE-A)	化膿性レンサ球菌	25,805	TSS，猩紅熱
(12)	SPE-B	〃	27,588	TSS，猩紅熱
(13)	SPE-C	〃	24,354	TSS，猩紅熱
(14)	YPM	エルシニア	21kDa	エルシニア感染症，泉熱
(15)	CPET	クロストリジウム	31kDa	腸炎
(16)	MAM	マイコプラズマ	10～15kDa	マウス関節炎

SEC_2の分子量34,000は精製外毒素よりの推定であり，実際はSEC_1，SEC_3と同程度と思われる．文献66)より．14)～16)は，藤巻，内山，生物科学45，125-131，1993より追加．

トキシックショック症候群(toxic shock syndrome：TSS)の原因毒素と考えられている．さらに，ブドウ球菌はリッター病(新生児剝脱性皮膚炎)や膿痂疹などの原因毒素であるexfoliative toxin(ET)*を産生するが，これもスーパー抗原として機能する．

レンサ球菌も猩紅熱の原因毒素と考えられているstreptococcal pyrogenic exotoxin(SPE：ディック毒あるいは発赤毒とも呼ばれる．A，

*exfoliative toxin(エキソホリアチン)：ブドウ球菌が産生する分子量約30,000のタンパク毒素で，血清型でAとBの二つの型がある．表皮を剝脱させる作用があり，リッター病の原因毒素．

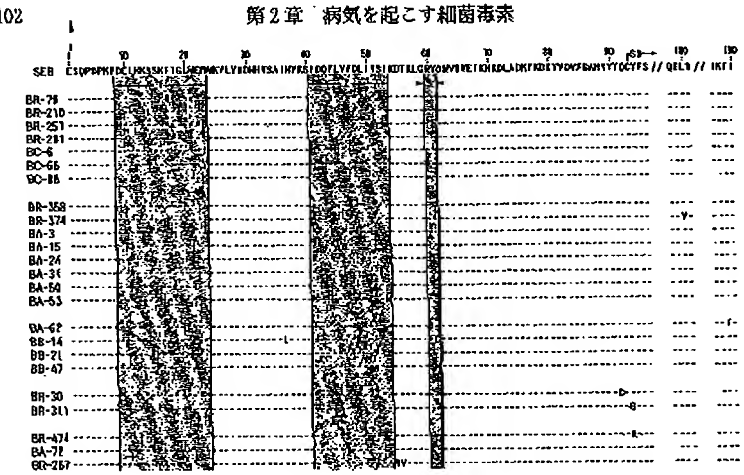

図2-39 ブドウ球菌エンテロトキシンB(SEB)の変異毒素を用いた機能解析
領域1はMHCIIおよびVβとの結合，領域2はMHCIIとの結合，領域3はVβとの結合に関与する．

図2-40 MHCクラスII分子の立体構造解明を伝える「Nature」誌 Vol. 364, No. 6432, 1993年．

B，Cに分類できる．最近Dも報告された)を産生し，スーパー抗原活性を発揮する．

SEのA～E間のみならずSPEもSEとアミノ酸配列上部分的な相同性が認められている．しかし，TSST-1には有意と思われる部分的な相同性は認められていない．

最近，SEB分子のどの構造部分がMHCクラスII分子やT細胞のVβ領域の結合に関与しているかを調べるため，SEの特定のアミノ酸を置換した変異毒素を用いた解析が行われている(図2-39)[67]．

また，一方で，MHCクラスII分子上にあるスーパー抗原の結合部位についても組み替え変異分子を用いた解析が行われている．MHCクラスII(図2-40)分子のα鎖がスーパー抗原の結合の主役を担っていると考えられているが，β鎖のHis80の結合への関与が示唆される，など詳細な事実が解明されつつある[68]．

一方，T細胞上のTCRがスーパー抗原をどのように認識しているかについても，組み替え遺伝子技術を応用して進められている．通常の抗原認識にかかわる部分とは異なる部位，つまりVβの外側でスーパー抗原を認識していることなどが明らかになってきている(図2-38参照)．

12.3 スーパー抗原の病原的意義

上に述べた種々なスーパー抗原活性を有する細菌毒素は，種々な細胞に対する直接的作用(たとえば，機能障害や致死・溶解作用)などを示さないことから，その作用のしくみについては，全くといってよいほど解明されていなかった．しかし，ここ数年，これらの毒素がスーパー抗原として作用し，T細胞を活性化させ，その結果，TNF-β，IL-2，INF-γらのリンホカインを出し，さらには単球やマクロファージの活性化もひき起こし，IL-1やTNF-αなどのモノカイン量を亢進させるなどの作用が明らかになってきた．適度のリンホカイン，モノカインは，感染防御機能を亢進させ，生体にとって有利な反応となると考えられるが，ときには，MHCクラスII分子をもつ種々なマクロファージ系細胞(肝クッパー細胞や皮膚ランゲルハンス細胞な

A part of translation of "Disease and Bacteriotoxin" (Takeshi HONDA, Bioscience Series, Published by Kagaku Dojin Ltd., January 25, 1994) p.101, Table 2-13

Table 2-13 Molecular weights and pathogenicities of extracellular toxins showing superantigen activity

Extracellular toxins	Origin	Molecular weight
(1) toxic shock syndorome toxin-1 (TSST-1)	Staphylococcal aureus	22049
(2) Staphylococcal enterotoxin A (SEA)	〃	27079
(3) SEB	〃	28494
(4) SEC_1	〃	27500
(5) SEC_2	〃	34000
(6) SEC_3	〃	27000
(7) SED	〃	27300
(8) SEE	〃	26425

Note: The molecular weight of SEC_2, 34000 is determined by estimate from purified exotoxin, therefore this is much the same as SEC_1 or SEC_3.
(SE=Staphylococcal enterotoxin)

A part of translation of "Disease and Bacteriotoxin" (Takeshi HONDA, Bioscience Series, Published by Kagaku Dojin Ltd., January 25, 1994) p.103, 1.3-5

SPE partly has homology of the amino acid sequence with any of SE species A-E, as one of the SE species has the homology with another one. However, It is not known that TSST-1 partly has homology with SE as assuming to be substantial.

SPECIFICATION

ENTEROTOXIN ADSORBENT, METHOD OF ADSORPTIVE REMOVAL, AND ADSORPTION APPARATUS

FIELD OF THE INVENTION

The present invention relates to an enterotoxin adsorbent, a method for adsorptive removal of an enterotoxin, and an adsorption apparatus comprising said adsorbent as packed in a housing.

BACKGROUND OF THE INVENTION

Enterotoxins are toxins produced by *Staphylococcus aureus*, among other bacteria, which have various biological activities such as emetic, pyrogenic and mitogenic activities, inducing symptoms of food poisoning or being causative of toxic shock syndrome (TSS).

Staphylococci are broadly distributed in the skin, nasal cavity, oral cavity, throat, urinary organs and intestinal canal of various animals inclusive of man as well as in the air, sewage water, river, foods and so forth and encompass a broad spectrum of species. Among such numerous species of staphylococci, the one pathogenic to human beings is *Staphylococcus aureus* (hereinafter referred to briefly as *S. aureus*) which is a coagulase-positive bacterium. *S. aureus* induces various infectious diseases and can be a causative factor in nosocomial infections, thus being of social concern.

As the enterotoxin produced by *S. aureus*, the following 10 species are known to this day: staphylococcal enterotoxins A, B, C1, C2, C3, D, E, G, H and I (hereinafter referred to briefly as SEA, SEB, SEC1, SEC2, SEC3, SED, SEE, SEG, SEH and SEI, respectively).

Enterotoxins are known to have superantigen activity. The ordinary antigen is taken up by the antigen-presenting cell and the antigen fragments available on fragmentation

(conversion to peptides of 10 to 15 amino acids) are presented, in the form bound to the pockets of MHC (major histocompatibility complex) class II molecule, on the surface of the antigen-presenting cell. These fragments are recognized by the TCR (T cell receptor) α- and β-chains of certain T cell clones, whereby the T cells are activated to set an immune reaction going. On the other hand, in the case of a superantigen, the antigen is not fragmented but directly bound to the MHC class II molecule on an antigen-presenting cell, and then the complex is recognized by TCR on the T cell to thereby activate the T cell. In this process, the antigen is recognized by the Vβ region of the TCR but unlike in the case of an ordinary antigen, the superantigen is recognized by substantially the entire population of T cells expressing the specific Vβ region to induce activation of the T cells and, hence, production of cytokines. Thus, in an individual exposed to a superantigen, an enormous population of T cells is activated as compared with the ordinary specific immune response to consequently release cytokines within a brief time, thus being suspected to induce abnormal reactions of the living body.

By using a specific antibody against an enterotoxin, an MHC class II protein or the like, the enterotoxin can be removed from a body fluid such as blood, plasma or serum, a culture supernatant, a foodstuff or a beverage but such antibodies are not only expensive but have the drawback that sterilization causes denaturation and serious decreases in adsorptive capacity.

Therefore, the advent has been awaited of an enterotoxin adsorbent which may be produced easily at low cost and will be highly effective.

Incidentally, Japanese Kokai Publication Hei-10-290833 discloses an adsorbent for TSST-1 (toxic shock syndrome toxin-1) comprising a compound having a log P (P denotes a partition coefficient in an octanol-water system) value of not less than 2.50 as immobilized but the literature is reticent

about adsorption of an enterotoxin.

SUMMARY OF THE INVENTION

The object of the present invention is to provide an adsorbent with which enterotoxins in body fluids can be efficiently adsorbed and removed, a method for adsorptive removal of an enterotoxin from a body fluid which comprises using said adsorbent, and an enterotoxin adsorption apparatus.

The inventors of the present invention explored in earnest for an adsorbent which may be capable of removing enterotoxins from body fluids with good efficiency. As a result, they discovered that the enterotoxin occurring in a body fluid can be efficiently adsorbed and removed with an adsorbent comprising a compound having a log P value of not less than 2.50 as immobilized on a water-insoluble carrier. The present invention has been developed on the basis of the above finding.

The present invention, therefore, is directed to an enterotoxin adsorbent comprising a compound with a log P, in which P represents a partition coefficient in an octanol-water system, value of not less than 2.50 as immobilized on a water-insoluble carrier.

The present invention is further directed to a method for adsorptive removal of an enterotoxin in a body fluid

which comprises contacting an enterotoxin-containing body fluid with an enterotoxin adsorbent,

said adsorbent comprising a compound with a log P, in which P represents a partition coefficient in an octanol-water system, value of not less than 2.50 as immobilized on a water-insoluble carrier.

The present invention is further directed to an enterotoxin adsorption apparatus

wherein a housing has an inlet and an outlet for a body fluid as well as a means for precluding flowing out of an adsorbent therefrom, and is packed therein an enterotoxin adsorbent,

said adsorbent comprising a compound with a log P, in which P represents a partition coefficient in an octanol-water system, value of not less than 2.50 as immobilized on a water-insoluble carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic cross-section view showing an enterotoxin adsorption apparatus embodying the principles of the invention.

Fig. 2 is a diagrammatic representation of the relation between flow rate and pressure loss as determined for 3 kinds of carrier materials.

EXPLANATION OF NUMERIC SYMBOLS

1. body fluid inlet
2. body fluid outlet
3. enterotoxin adsorbent
4. and 5. filter which allows passage of a body fluid but does not allow passage of said enterotoxin adsorbent
6. column
7. enterotoxin adsorption apparatus

DETAILED DESCRIPTION OF THE INVENTION

The enterotoxin in the context of the present invention is a toxin comprising a soluble protein having a molecular weight of 25,000 to 30,000 as produced by S. aureus.

The body fluid includes blood, plasma, serum, ascites, lympha, synovial fluid, fractions or components of any of them, and other humoral biological materials.

The log P value is a parameter of hydrophobicity of a compound and the partition coefficient P in the representative octanol-water system is determined in the following manner. Thus, the compound of interest is first dissolved in octanol (or water), an equal quantity of water (or octanol) is then added, and the mixture is shaken with Griffin flask shaker

(manufactured by Griffin & George, Ltd.) for 30 minutes. The mixture is then centrifuged at 2000 rpm for 1 to 2 hours and the concentrations of the compound in the octanol layer and the water layer are measured spectrometrically or by GLC at room temperature and atmospheric pressure, among other techniques. Then, the P value is calculated by means of the following equation.

P=Coct/Cw
Coct: concentration of the compound in octanol layer
Cw: concentration of the compound in water layer

The adsorbent of the invention comprises a water-insoluble carrier and, as supported thereon, a compound having a log P value of not less than 2.50 as determined by the above method.

The log P values of various compounds have so far been measured by many workers and such measured log P values have been compiled by C. Hansch et al. Partition Coefficients and Their Uses; Chemical Reviews 71, 525 (1971) .

Referring to compounds with unknown measured log P values, the values ($\sum f$) calculated using the hydrophobic fragmental constant f shown in The Hydrophobic Fragmental Constant, Elsevier Sci. Pub. Com., Amsterdam (1977) can be used as a reference. The hydrophobic fragmental constant is a value representing the hydrophobicity of various fragments as determined statistically based on a large number of measured log P values. The sum of f values of various fragments constituting a compound is approximately equal to the log P value. The term log P as used in this invention for any compound with an unknown log P value means the $\sum f$ value of the compound.

In the screening for compounds effective for adsorption of enterotoxins, compounds having various log P values were respectively immobilized on a water-insoluble carrier and evaluated for their adsorptive affinity for enterotoxins. As

a result, it was found that compounds having log P values not smaller than 2.50, preferably not smaller than 2.80, more preferably not smaller than 3.00, are effective in the adsorption of enterotoxins, while compounds with log P values smaller than 2.50 do not appreciably adsorb enterotoxins. By way of illustration, assuming that an alkylamine is immobilized as said compound on a water-insoluble carrier, the change from n-hexylamine (log P = 2.06) to n-octylamine (log P = 2.90) leads to a phenomenal increase in the adsorptive affinity for enterotoxins. These findings suggest that the adsorption of an enterotoxin by the adsorbent of the invention is attributable to the hydrophobic interaction between the enterotoxin and the atomic group introduced onto the carrier by immobilization of a compound having a log P value of not less than 2.50 and that any compound having a log P value of less than 2.50 is too low in hydrophobicity to exhibit a sufficiently high adsorptive affinity for enterotoxins.

The compound that can be immobilized on a water-insoluble carrier with advantage in the practice of the invention is not particularly restricted only provided that its log P value is not smaller than 2.50. However, when a compound is immobilized on a carrier by a chemical coupling reaction, a portion of the compound is eliminated in many instances and in case the eliminated group contributes a great deal to the overall hydrophobicity of the compound, that is to say the hydrophobicity of the atomic group immobilized on the carrier is reduced to a $\sum f$ value of less than 2.50 due to the eliminated group, the particular compound is not suitable in light of the object and spirit of the invention. A case in point is the immobilization of isopentyl benzoate ($\sum f = 4.15$) on a hydroxyl group-containing carrier by a transesterification reaction. In this case, the atomic group actually immobilized on the carrier is C_6H_5CO- and this atomic group has a $\sum f$ value of less than 1. Whether a compound of this type is suitable as the compound for use in the invention can be simply found by checking

to see whether the compound obtainable by substituting hydrogen for the eliminated group has a log P value of not less than 2.50.

The preferred, among compounds having log P values not smaller than 2.50, are unsaturated hydrocarbons, alcohols, amines, thiols, carboxylic acids and derivatives thereof, halides, aldehydes, hydrazides, isocyanates, oxirane ring-containing compounds such as glycidyl ethers, halogenated silanes, and other compounds having functional groups useful for bonding to the carrier. As representative examples of such compounds, there can be mentioned amines such as n-heptylamine, n-octylamine, decylamine, dodecylamine, hexadecylamine, octadecylamine, 2-aminooctene, naphthylamine, phenyl-n-propylamine, diphenylmethylamine, etc.; alcohols such as n-heptyl alcohol, n-octyl alcohol, dodecyl alcohol, hexadecyl alcohol, 1-octen-3-ol, naphthol, diphenylmethanol, 4-phenyl-2-butanol, etc. and glycidyl ethers of such alcohols; carboxylic acids such as n-octanoic acid, nonanoic acid, 2-nonenoic acid, decanoic acid, dodecanoic acid, stearic acid, arachidonic acid, oleic acid, diphenylacetic acid, phenylpropionic acid, etc. and the corresponding acid halides and derivatives such as esters and amides; halides such as octyl chloride, octyl bromide, decyl chloride, dodecyl chloride, etc.; thiols such as octanethiol, dodecanethiol, etc.; halosilanes such as n-octyltrichlorosilane, octadecyltrichlorosilane, etc., and aldehydes such as n-octyl aldehyde, n-capryl aldehyde, dodecyl aldehyde, and so forth.

Aside from the foregoing compounds, compounds such that the hydrogen atom in the hydrocarbon moiety of any of the compounds mentioned by way of example above have been substituted by a halogen atom, a substituent group containing a hetero atom such as N, O or S, a different alkyl group or the like and having log P values not smaller than 2.50, as well as compounds having log P values not less than 2.50 among the compounds listed in the above general review of C. Hansch et al. Partition Coefficients and Their Uses: Chemical Reviews

71, 525 (1971), pages 555~613 can also be mentioned. For use in the invention, these compounds are not exclusive choices.

These compounds may be used each independently or in a combination of two or more species, optionally even in combination with a compound having a log P value smaller than 2.50.

The water-insoluble carrier as a constituent of the adsorbent of the invention is a material which is solid at atmospheric temperature and pressure and only sparingly soluble in water.

The shape of the water-soluble carrier in the present invention may for example be granular, sheet-like, filamentous or hollow fiber-like, although these are not exclusive choices. The size of the carrier is not particularly restricted, either.

The water-insoluble carrier as a constituent of the adsorbent of the invention includes inorganic matrices such as glass beads, silica gel, etc.; organic matrices such as synthetic polymers, e.g. crosslinked polyvinyl alcohol, crosslinked polyacrylates, crosslinked polyacrylamide, crosslinked polystyrene, etc. and polysaccharides, e.g. crystalline cellulose, crosslinked cellulose, crosslinked agarose, crosslinked dextrin, etc.; and composite matrices comprising organic-organic, organic-inorganic, or other combinations of the above-mentioned matrices, to mention typical examples.

Among these, hydrophilic matrices are preferred because of their comparatively low nonspecific adsorption and good adsorption selectivity for enterotoxins. The term "hydrophilic carrier" is used herein to mean a carrier such that when the compound constituting the carrier is made into a flat plate, the angle of contact between it and water is not greater than 60 degrees. Various techniques are known for measurement of the contact angle of water but as described by Ikeda in his book Jikken Kagaku Sensho (Selected readings in Experimental Chemistry), Chemistry of Colloids, Chapter 4, Thermodynamics

of Interfaces, pp. 75 to 104, Mokabo (1986), the most common method comprises placing a drop of water on a flat plate made of the compound and measuring the angle of contact with water. The compound giving such an angle of contact not greater than 60 degrees as measured by the above method includes cellulose, polyvinyl alcohol, saponified ethylene-vinyl acetate copolymer, polyacrylamide, polyacrylic acid, polymethacrylic acid, poly(methyl methacrylate), polyacrylic acid-grafted polyethylene, polyacrylamide-grafted polyethylene, glass and so forth.

These water-insoluble carrier materials preferably have a multiplicity of pores of suitable size, that is to say a porous structure. The carrier having a porous structure includes not only a carrier having spaces (macropores) defined by clusters of microspheres when a basal carrier polymer forms single spherical particles by cohesion of microspheres but also a carrier having micropores formed among the clusters of cores within each microsphere constituting a basal polymer carrier and a carrier having micropores formed when a copolymer having a three-dimensional structure (a polymer network) is swollen in the presence of an organic solvent having an affinity for the polymer.

Furthermore, in consideration of the adsorptive capacity per unit volume of the adsorbent, said water-insoluble carrier having a porous structure is more preferably of the total porosity type than of the surface porosity type and the void volume and specific surface area are preferably as large as possible within limits not detracting from adsorption efficiency.

As a carrier satisfying these preferred requirements, a porous cellulose carrier can be mentioned. The porous cellulose carrier has several meritorious characteristics. Thus, (1) because it has comparatively high mechanical strength and toughness, this carrier does not collapse or give dust in stirring and other operations and even when a body fluid is

passed through a column packed with the carrier at a high speed, the carrier is not compacted, thus permitting a high flow rate. Furthermore, the porous structure of the carrier is not easily affected by high-pressure steam sterilization. (2) Because this carrier is made up of cellulose, it is hydrophilic, has a large number of hydroxyl groups available for binding a ligand, and features little nonspecific adsorption. (3) Even if the void volume is increased, an adsorptive capacity as large as that of a soft carrier may be insured because of its comparatively high strength. (4) The carrier ranks high in safety as compared with synthetic polymer and other matrices. Therefore, this carrier is one of the most favorable matrices for use in the present invention, although this is not an exclusive choice. Moreover, the above-mentioned matrices may be used each independently or in the form of a mixture of two or more species.

More preferably, said water-insoluble carrier having a porous structure is characterized in that while the adsorption load may enter its pores with a fairly high probability, the entry of other proteins is precluded as much as possible. Thus, the enterotoxin to be adsorbed by the adsorbent of the invention is a protein having a molecular weight within the range of 25,000 to 30,000 and for efficient adsorption of this protein, the carrier is preferably such that the enterotoxin may find its way into its porous structure in a large ratio but other proteins are prevented from entering the pores. As a molecular weight marker of a substance capable of entering a porous structure, the molecular weight of exclusion limit is generally used. As described in several books (e.g. Hatano Hiroyuki & Hanai Toshihiko: Experimental high Performance Liquid Chromatography, Kagaku Dojin), the molecular weight of exclusion limit means the molecular weight of the smallest of the molecules prevented from entering the pores (entry rejected) in gel permeation chromatography. Molecular weights of exclusion limit have been well documented generally for

globular proteins, dextran, polyethylene glycol, etc. and in the case of the carrier for use in the invention, it is appropriate to use the value found for globular proteins.

Investigations undertaken by the inventors using matrices varying in molecular weight of exclusion limit revealed that the range of molecular weights of exclusion limit for globular proteins which is suitable for adsorption of enterotoxins is 5,000 to 600,000. Thus, when a carrier having a molecular weight of exclusion limit of less than 5000 for globular protein is employed, the amount of adsorption of enterotoxins is too small to endorse its practical utility. On the other hand, when the molecular weight of exclusion limit of 600,000 is exceeded, the adsorption of proteins (mostly albumin) other than enterotoxins is increased so that the practical utility of the carrier is low in terms of selectivity. Therefore, the preferred range of molecular weights of exclusion limit for globular protein as the carrier in the present invention is 5,000 to 600,000, with the range of 6,000 to 400,000 being the more preferred and the range of 10,000 to 300,000 being particularly preferred.

Furthermore, the carrier preferably has a functional group which can be used for the ligand-binding reaction. The functional group mentioned above includes but is not limited to hydroxyl, amino, aldehyde, carboxyl, thiol, silanol, amido, epoxy, halogen, succinylimino, and acid anhydride.

The carrier which can be used in the present invention includes both a rigid carrier and a soft carrier. However, when it is used as a constituent of the adsorbent for extracorporeal circulation, it is important that when the adsorbent is packed into a column and the body fluid is passed through it, no plugging should take place. To ensure this, a sufficient mechanical strength is required of the carrier. Therefore, the carrier for use in the invention is more preferably a rigid carrier. The rigid carrier mentioned above means a carrier such that, taking a granular carrier as an example, when a cylindrical

column is evenly packed with the carrier and an aqueous fluid is passed, the relation between pressure loss ΔP and flow rate is linear up to 0.3 kg/cm^2 as described hereinafter in Reference Example.

While the adsorbent of the invention can be obtained by immobilizing a compound having a log P value of not less than 2.50 on a water-insoluble porous carrier, various known techniques can be liberally used for the immobilization. However, when the adsorbent of the invention is used for extracorporeal circulation therapy, it is important from safety points of view to minimize the risk of elimination and elution of the ligand during sterilization and therapy and, in this sense, immobilization by covalent bonding is preferred.

Various techniques are available for the adsorptive removal of an enterotoxin from body fluids by means of the adsorbent of the invention. The simplest method comprises withdrawing a body fluid into a bag or the like, mixing the adsorbent with the body fluid to adsorptively remove enterotoxins, and filtering off the adsorbent to recover the fluid having eliminated the enterotoxins. Another method comprises packing the adsorbent into a housing equipped with an inlet and an outlet for a body fluid and, disposed at the outlet, further with a filter which allows passage of a body fluid but intercepts the adsorbent and passing the body fluid through the housing. Whichever desired of the methods can be utilized but the latter method is not only expedient but, when the system is built into an extracorporeal circuit, enables one to remove enterotoxins from a patient's body fluid, particularly the blood, on line and with good efficiency. The adsorbent of the invention is suitable for this method.

In the extracorporeal circuit mentioned above, the adsorbent of the invention may be used independently but may be used in combination with a different extracorporeal therapeutic system. As an example of such combination, an artificial dialysis circuit can be mentioned. Thus, the

adsorbent can be used in conjunction with a dialysis treatment.

The enterotoxin adsorption apparatus of the invention, which makes use of the above-described adsorbent of the invention, is now described with reference to Fig. 1 which is a schematic cross-section view showing an embodiment. In Fig. 1, the reference numeral 1 represents a body fluid inlet, 2 a body fluid outlet, 3 an enterotoxin adsorbent according to the invention, 4 and 5 each a filter which allows passage of the body fluid and its components but does not allow passage of said enterotoxin adsorbent, 6 a column, and 7 an enterotoxin adsorption apparatus. It should, however, be understood that the enterotoxin adsorption apparatus is not restricted to such a specific embodiment but may be any apparatus comprising a housing equipped with an inlet and an outlet for a fluid and a means for precluding flowing out of an enterotoxin adsorbent therefrom and, as packed therein, said enterotoxin adsorbent.

The means for precluding flowing out of the adsorbent includes a wire-mesh filter, a nonwoven cloth filter, a cotton pad filter, and so forth. The housing is not particularly restricted in geometry, material or size but one having a columnar configuration is preferred. The preferred housing material withstands a sterilization procedure, thus including silicon-coated glass, polypropylene, poly(vinyl chloride), polycarbonate, polysulfone, polymethylpentene, and so forth. The capacity and size of the housing are preferably 50 to 1500 ml and 2 to 20 cm in diameter, more preferably 100 to 800 ml and 3 to 15 cm in diameter, still more preferably 150 to 400 ml and 4 to 10 cm in diameter.

By means of the adsorbent comprising a compound having a log P value of not less than 2.50 as immobilized on a water-insoluble carrier according to the invention, enterotoxins can be adsorbed and removed from body fluids with good efficiency.

EXAMPLES

The following examples are intended to describe the present invention in further detail and should by no means be construed as defining the scope of the invention.

Reference Example

A glass-made cylindrical column (9 mm in. dia. × 150 mm long) fitted with a 15 μm (pore size) filter at both ends was uniformly filled with agarose material (product of Bio-Rad; Biogel A-5m, particle diameter 50 to 100 mesh), vinyl polymer material (product of Tosoh Corporation; Toyopearl HW-65, particle diameter 50 to 100 μm) and cellulose material (product of Chisso Corporation; Cellulofine GC-700m, particle diameter 45 to 105 μm). Using a peristaltic pump, water was caused to flow through the column and the relation between flow rate and pressure loss ΔP was determined. The result is shown in Fig. 2.

It is apparent from Fig. 2 that whereas the flow rate increased in substantial proportion with an increasing pressure in the cases of Toyopearl HW-65 and Cellulofine GC-700m, the flow rate failed to increase owing to compaction even when the pressure was increased in the case of Biogel A-5m. For the purposes of the present invention, any carrier, such as the former two materials, which gives a linear relation between pressure loss ΔP and flow rate up to 0.3 kg/cm^2 is defined as a rigid carrier.

Example 1

To 170 ml of the cellulosic porous carrier Cellulofine GC-200m (product of Chisso Corporation; molecular weight of exclusion limit for globular protein: 140,000) was added sufficient water to make 340 ml. Then, 90 ml of 2 M sodium hydroxide/H_2O was added and the temperature was adjusted to 40℃. To this was added 31 ml of epichlorohydrin, and the reaction was carried out at 40 ℃ with stirring for 2 hours. After

completion of the reaction, the reaction product was rinsed well with water to give epoxidized Cellulofine GC-200m.

To 10 ml of this epoxidized Cellulofine GC-200m was added 200 mg of n-hexadecylamine ($\Sigma f = 7.22$), and the reaction was conducted in ethanol under stationary conditions at 45 ℃ for 6 days. After completion of the reaction, the reaction product was washed thoroughly with ethanol and water in the order mentioned to give n-hexadecylamine-immobilized Cellulofine GC-200m.

To 0.5 ml of the above n-hexadecylamine-immobilized Cellulofine GC-200m was added 3 ml of fetal bovine serum (FBS) containing about 600 pg/ml each of 3 different enterotoxins, namely SEA, SEB, and SEC1, and the mixture was shaken at 37 ℃ for 2 hours. After 2 hours, the adsorbent was separated from the supernatant and the concentrations of the respective enterotoxins in the supernatant were determined by ELISA.

The ELISA of each enterotoxin was carried out as follows. The primary antibody rabbit anti-SEA (or SEB or SEC) IgG (product of Toxin Technology) was diluted with a coating buffer and distributed onto a microtiter plate, 100 µl per well. After overnight standing at 4 ℃, the microtiter plate was washed and 3% bovine serum albumin solution was added, 200 µl per well. The plate was allowed to sit again at room temperature for 2 hours and, then, washed. Thereafter, the standard solutions of the respective enterotoxins and the supernatants before and after incubation were placed in a 100 µl microtiter plate. After 2 hours of sitting at room temperature, the plate was washed. The secondary antibody rabbit anti-SEA (or SEB or SEC) HRP (product of Toxin Technology) was diluted with 1% bovine serum albumin solution and added, 100 µl/well. After 2 hours of sitting at room temperature, the plate was washed. Then, 100 µl/well of o-phenylenediamine solution was added and the plate was allowed to sit at room temperature for 10 minutes. Thereafter, 100 µl/well of 4 N-sulfuric acid was added and the absorbance at 492 nm was measured. By comparison with the

absorbance of the standard solution, the concentration of each enterotoxin was estimated.

Example 2

To 10 ml of the epoxidized Cellulofine GC200m obtained in Example 1 was added 200 mg of n-octylamine (log P = 2.90), and the reaction was conducted in 50 (v/v) % ethanol/water at 45℃ under stationary conditions for 6 days. After completion of the reaction, the reaction product was washed thoroughly with 50 (v/v) % ethanol/water, ethanol, 50% (v/v) % ethanol/water, and water in the order mentioned to give n-octylamine-immobilized Cellulofine GC200m.

The above n-octylamine-immobilized Cellulofine GC200m was shaken with FBS containing 3 kinds of enterotoxins just as in Example 1. The adsorbent was separated from the supernatant and the concentration of each enterotoxin in the supernatant was determined by ELISA.

Comparative Example 1

Using n-hexylamine (log P = 2.06) in lieu of n-octylamine, the procedure of Example 2 was otherwise faithfully repeated to give n-hexylamine-immobilized Cellulofine GC200m. This n-hexylamine-immobilized Cellulofine GC200m was shaken with FBS containing 3 kinds of enterotoxins just as in Example 1. The adsorbent was separated from the supernatant and the concentration of each enterotoxin in the supernatant was determined by ELISA.

Comparative Example 2

Using n-butylamine (log P = 0.97) in lieu of n-octylamine, the procedure of Example 2 was otherwise faithfully repeated to give n-butylamine-immobilized Cellulofine GC200m. This n-butylamine-immobilized Cellulofine GC200m was shaken with FBS containing 3 kinds of enterotoxins just as in Example 1. The adsorbent was separated from the supernatant and the

concentration of each enterotoxin in the supernatant was determined by ELISA.

Comparative Example 3

Cellulofine GC-200m was shaken with FBS containing 3 kinds of enterotoxins as in Example 1. The adsorbent was separated from the supernatant and the concentration of each enterotoxin in the supernatant was determined by ELISA.

Table 1

	Concentration of enterotoxin (pg/ml)		
	SEA	SEB	SEC1
Example 1	150	130	180
Example 2	200	150	210
Comp. Ex. 1	565	590	500
Comp. Ex. 2	570	600	520
Comp. Ex. 3	580	610	540

CLAIMS

1. An enterotoxin adsorbent comprising a compound with a log P, in which P represents a partition coefficient in an octanol-water system, value of not less than 2.50 as immobilized on a water-insoluble carrier.

2. The adsorbent according to Claim 1 wherein said water-insoluble carrier is a water-insoluble porous carrier.

3. The adsorbent according to Claim 2 wherein said water-insoluble porous carrier has a molecular weight of exclusion limit of 5000 to 600000 for globular protein.

4. A method for adsorptive removal of an enterotoxin in a body fluid which comprises contacting an enterotoxin-containing body fluid with the adsorbent according to Claim 1.

5. An enterotoxin adsorption apparatus wherein a housing has an inlet and an outlet for a body fluid as well as a means for precluding flowing out of an adsorbent therefrom, and is packed therein the adsorbent according to Claim 1.

ABSTRACT

The present invention is directed to an enterotoxin adsorbent comprising a compound having a log P (P denotes a partition coefficient in an octanol-water system) value of not less than 2.50 as immobilized on a water-insoluble carrier.

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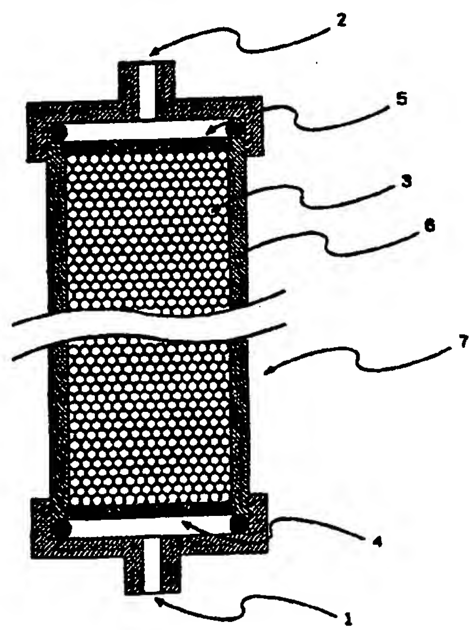
(54) Title : ADSORBENT FOR ENDOTOXIN, TUMOR NECROSIS FACTOR-α OR INTERLEUKINS, METHOD FOR REMOVAL VIA ADSORPTION, AND ADSORBER

(54) 発明の名称　エンドトキシン、腫瘍壊死因子-α、またはインターロイキン類の吸着剤、吸着除去方法および吸着器

(57) Abstract

An adsorbent which comprises a styrene/divinylbenzene copolymer having sulfonate groups and is used for endotoxins, tumor necrosis factor-α (TNF-α) or interleukins; a method for removing endotoxins, TNF-α or interleukins comprising the step of bringing the adsorbent into contact with a liquid containing endotoxins, TNF-α or interleukins; and an adsorber for endotoxins, TNF-α or interleukins composed of the adsorbent packed in a container having an inlet and an outlet.



(57) 要約

スルホン酸基を有するスチレンージビニルベンゼン共重合体から構成される、エンドトキシン、腫瘍壊死因子－α（TNF－α）、またはインターロイキン類の吸着剤、および、該吸着剤と、エンドトキシン、TNF－α、またはインターロイキン類を含有する液体とを接触させる工程を包含するエンドトキシン、TNF－α、またはインターロイキン類の吸着除去方法が提供される。該吸着剤を入口と出口を有する容器に充填したエンドトキシン、TNF－α、またはインターロイキン類の吸着器もまた提供される。

明細書

エンドトキシン、腫瘍壊死因子－α、またはインターロイキン類の吸着剤、吸着除去方法および吸着器

技術分野

本発明は、エンドトキシン、腫瘍壊死因子－α（ＴＮＦ－α）、およびインターロイキン類（インターロイキン－１β（ＩＬ－１β）、インターロイキン－２（ＩＬ－２）、インターロイキン－６（ＩＬ－６）、およびインターロイキン－８（ＩＬ－８）から選択される物質をいう）からなる群より選択される少なくとも１種の物質の吸着剤、該吸着剤を用いた該物質の吸着除去方法、ならびに該物質の吸着器に関する。

背景技術

細菌感染から炎症を引き起こす代表的な物質として、グラム陰性菌の表面に存在するエンドトキシンが知られている。このエンドトキシンの作用により、種々のサイトカインや活性化補体の産生が促進される。実際、細菌感染による炎症を支配する物質は、これらのサイトカインや活性化補体である。これらサイトカインの中ではＴＮＦ－αが最も重要視されている。

例えば、敗血症では、細菌感染によりエンドトキシンが産生され、全身的な炎症反応を起こす。この炎症症状が亢進し